PATENT
ATTORNEY DOCKET NO.: INGA,002/C/CIP

APPLICATION FOR UNITED STATES LETTERS PATENT

Entitled

IN VIVO PRODUCTION OF ssDNA CONTAINING DNA ENZYME SEQUENCE WITH RNASE ACTIVITY

Applicant: Charles A. Conrad
Overland Park, Kansas

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Number: EE874591973US
Date of Deposit: October 4, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and is addressed to Box Patent Application, The Honorable Commissioner of Patent and Trademarks, Washington, D.C. 20231.

Mark R. Wisner, Registration No. 30,603 — October 4, 1999 (Date of Signature)

IN VIVO PRODUCTION OF ssDNA CONTAINING DNA ENZYME SEQUENCE WITH RNASE ACTIVITY

BACKGROUND OF THE INVENTION

SUB A1

This application is a continuation-in-part of my co-pending application Serial No. 09/397,782, entitled IN VIVO PRODUCTION OF ssDNA USING REVERSE TRANSCRIPTASE WITH PREDEFINED REACTION TERMINATION VIA STEM-LOOP FORMATION, filed September 16, 1999, and co-pending application Serial No. 09/169,793, entitled PRODUCTION OF ssDNA *IN VIVO*, filed October 9, 1998. Both Serial Nos. 09/169,793 and 09/397,782 are continuations-in-part of application Serial No. 08/877,251, entitled STEM-LOOP CLONING VECTOR AND METHOD, filed June 17, 1997. Serial No. 08/877,251 is a continuation application of application Serial No. 08/236,504, having the same title, filed April 29, 1994.

The present invention relates to production of single-stranded DNA (ssDNA) from a plasmid-based system within eukaryotic cells. More specifically, the present invention relates to methods and compositions for expression of any desired ssDNA sequence including a sequence capable of performing unique ezymatic reactions within eukaryotic host cells without contiguous plasmid vector sequences. The vector system of the present invention removes contiguous plasmid vector sequences either by stem-loop formation with subsequent premature termination of a reverse transcription reaction or by cleavage of stem-loop intermediates. The ssDNA that is produced by this method can be designed so that it is complimentary to any endogenous nucleic acid sequence target.

So far as is known, there is no method for producing single-stranded deoxyribonucleic acid (ssDNA) species in eukaryotic cells which do not contain intervening and/or flanking vector sequences. The scientific and patent literature does include the disclosure of cDNA-producing vectors (*see* A. Ohshima, *et al.*, 89 Proc. Natl. Acad. Sci. USA 1016-1020 (1992); S. Inouye, *et al.*, 3 Current Opin. Genet. Develop. 713-718 (1993); O. Mirochnitchenko, *et al.*, 269 J. Biol. Chem. 2380-2383 (1994); J.-R. Mao, *et al.*, 270 J. Biol. Chem. 19684-19687 (1995); and U.S. Patent Nos. 5,436,141 and 5,714,323), but the systems disclosed in these references do not appear to have

demonstrated the ability to produce ssDNA in eukaryotic cells without intervening vector sequences. Instead, the ssDNA produced by the method described in these references includes intervening nucleotide sequences which can interfere with the intended function of the ssDNA product.

There are a number of naturally occurring biological elements that employ an ssDNA intermediate within their life cycles. Viral and transposable elements that have been discovered which contain ssDNA intermediates within their life cycles in eukaryotic and prokaryotic systems, as well as yeast (*see* A.M. Weiner, *et al.*, 55 Ann. Rev. Biochem. 631-661 (1986) and H. Varmus, *et al.*, *in* Mobile DNA, M.M. Howe and D.E. Berg (Eds.), American Society for Microbiology: Washington, D.C., pp. 53-108 (1989)). Many of these genetic elements can be adapted to produce single-stranded nucleic acids within eukaryotic systems, but the nucleic acids produced from such elements would necessarily contain genetic information which could interfere with the desired function of the *in vivo* produced single-stranded oligonucleotides. Further, such elements are difficult to manipulate beyond their normal biological life cycles to carry desired nucleotide sequences into the cell. The literature also describes RNA expression vectors that produce antisense RNA in cells, but the RNA:RNA hybrids which are formed are not as stable as DNA:RNA hybrids, the synthesized RNA has a shorter half-life than DNA, and RNA-RNA hybrids do not stimulate RNase H activity, which may be critical for *in vivo* antisense efficacy, as does the DNA:RNA hybrids (H. Donis-Keller, 7 Nucleic Acids Res. 179-192 (1979)). Further, as will be made apparent herein, the present invention makes advantageous use of the enzymatic activity of certain ssDNA. Because RNA sequences do not show such activity, they are unsuitable for use in connection with the present invention.

Artificially synthesized DNA analog oligomers used for antisense therapies must be administered intravenously, which involves problems in cell uptake and distribution (P.A. Cossum, *et al.*, 267 J. Pharmacol. Expl. Ther. 1181-1190 (1993); H. Sands, *et al.*, 47 Mol. Pharmacol. 636-646 (1995)) as well as toxicity problems due to the high blood concentrations required to be effective (S.P. Henry, *et al.*, 116 Toxicology 77-88 (1997)). By far the most used DNA analogs in antisense therapies are phosphorothioates and methylphosphonates. However, phosphorothioate oligonucleotides tend to bind serum

and intracellular proteins nonspecifically (S.T. Crooke, *et al.*, 227 J. Pharmacol. Exp. Ther. 923-937 (1996); W.Y. Gao, *et al.*, 41 Mol. Pharmacol. 223-229 (1992)), and at higher concentrations, they inhibit RNase H activity (S.T. Crooke, *et al.*, 312 Biochem. J. 599-608 (1995)). Phosphorothioate oligonucleotides have a lower Tm (an average of 0.50C per base-pair) for RNA than does DNA (S.T. Crooke, *et al.*, Antisense Research and Application, CRC Press: Boca Raton (1993)), which requires that phosphorothioate oligonucleotides be typically longer than phoshodiester DNA oligonucleotides for effective binding, which can cause a loss of hybridization specificity (J.-J. Toulmé, *et al.*, *in* C. Lichtenstein and W. Nellen (Eds.), Antisense Technology: A Practical Approach IRL Press: New York, pp. 39-74 (1997)). Further, although their pharmacokinetic properties appear to be more favorable than unmodified oligonucleotides, the half life of phosphorothioate oligonucleotides in many experimental animals is less than one hour (S. Agrawal, *et al.*, 88 Proc. Natl. Acad. Sci. USA 7595-7599 (1991); P. Iverson, 6 Anticancer Drug Design 531-538 (1991)) such that delivery of an efficacious dose of the oligonucleotide may be problematical. Methylphosphonate oligonucleotides do not activate RNase H enzyme activity (L.J. Maher, *et al.*, 245 Science 725-730 (1989); P.S. Miller, *in* J.S. Cohen (Ed.), Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression, CRC Press: Boca Raton, p, 79 (1989)) and are eliminated rapidly (T.L. Chen, *et al.*, 18 Drug Metab. Dispos. Biol. Fate. Chem. 815 (1990)).

Another factor influencing the delivery of synthetic oligonucleotides into cells is the low permeability of the cell membrane to such compounds. Low permeability may effectively preclude adequate uptake for sustained *in vivo* activity. Consequently, many oligonucleotides must be delivered to the cell by carrier systems such as liposomes or molecular complexing agents. However, the relatively short circulating time of liposomes, lack of targeting specificity, and the need for repeated exposure severely limits the usefulness of synthetic oligonucleotide delivery schemes. These difficulties can be avoided, however, if production of the desired single-stranded oligonucleotide takes place within the cell (*in vivo*). Nevertheless, so far as is known, no method for producing single-stranded nucleic acid in eukaryotic cells is available which overcomes all of these limitations and disadvantages.

The patent and scientific literature also discloses the existence of short deoxynucleic acid sequences that have been shown to have catalytic activity (*see*, Breaker, R.R. and G.F. Joyce, 1 Chem. Biol. 223-229 (1994); Cuenoud, B. and J.W. Szostak, 375 Nature 611-613 (1995); Santoro, S.W. and G.F. Joyce, 94 Proc. Natl. Acad. Sci. USA 4262-4266 (1997); Faulhammer and M. Famulok, 269 J. Molec. Bio. 188-203 (1997); Carmi, N, *et al.*, 95 Proc. Natl. Acad. Sci. USA (1998); Li, Y. and R.R. Breaker, 96 Proc. Natl. Acad. Sci. USA 2746-2751 (1999) and U.S. Patent Nos. 5,807,718 and 5,910,408), including the so-called "10-23 DNA enzyme" and other ssDNA sequences that act, for instance, as copper-dependent DNA ligases and calcium-dependent DNA kinases. The catalytic efficiency of such sequences has been demonstrated for cleaving mRNA targets at 10^9 m^{-1}/min^{-1} in the presence of divalent magnesium, thereby offering the opportunity for targeted destruction of substrate molecules (*see, for instance,* R.R. Breaker, *supra*). Although the art appears to recognize the potential for use of this enzymatic activity for therapeutic purposes, so far as is known, no system is available for producing these target-specific enzymatic nucleic acid sequences to produce a therapeutic effect *in vivo*.

There are, therefore, no systems available that utilize the potential advantage of the efficient catalytic activity of these enzymatic nucleic acid sequences. It is, therefore, an object of the present invention to provide DNA constructs, and methods of using such constructs, which overcome these limitations and disadvantages. In more detail, it is an object of the present invention to provide a DNA construct that directs the synthesis of ssDNA containing a sequence that specifically cleaves mRNA targets *in vivo*.

Because secondary structure folding is critical to the catalytic function of the "DNA enzyme" sequence of the ssDNA, it is another object of the present invention to provide methods, and DNA constructs, for producing ssDNA including such a DNA enzyme sequence of any desired nucleotide sequence within eukaryotic cells without undesirable intervening or flanking nucleotide bases.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA *in vivo* in a manner which isolates or presents a sequence of interest to the desired target without interference from intervening or flanking nucleotide bases.

Another object of the present invention is to provide a method, and a DNA construct utilized in such methods, for production of ssDNA within eukaryotic cells which contains DNA enzyme sequences for overcoming the significant problems encountered by the use of standard oligonucleotide delivery methods for therapeutic purposes.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA of any nucleotide sequence *in vivo* which can be used as (but is not limited to) an inhibitory nucleic acid for, for instance, binding to mRNA in an anti-sense fashion to down regulate a gene product or a viral gene product of interest or binding to and inhibiting a specific cellular function, for instance, by binding to proteins which recognize a nucleic acid sequence.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA designed in such a way as to favor binding to duplex (native DNA) to form triplex structures which may interfere with normal gene transcription and regulation.

Another object of the present invention is to produce ssDNA within eukaryotic cells for the purpose of disrupting one or more highly regulated cell functions.

Yet another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA into which secondary structures are designed so that the ssDNA oligonucleotides bind to and/or otherwise inhibit or activate various cellular functions which rely on nucleic acid protein interaction such as transcription, translation, and DNA replication.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA *in vivo* for site-directed mutagenesis or gene knockout for therapeutic applications.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA of precisely defined nucleotide composition which favors site-specific insertion into a genome for therapeutic purposes.

Yet another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA that is complimentary to any endogenous nucleic acid sequence target.

SUB A2

Still another object of the present invention is to provide a unique ss-cDNA "stem-loop" structure that is designed so that the stem is comprised of an inverted tandem repeat that folds back on itself to form a stable, double-stranded stem that terminates reverse transcriptase products, thereby eliminating undesirable 5' sequence portions of the intermediate RNA transcript needed for ssDNA formation *in vivo*.

Another object of the present invention is to provide a method, and a DNA plasmid utilized in such a method, for *in vivo* production of ssDNA including a sequence exhibiting catalytic activity against mRNA targets for transfection into eukaryotic cells which overcomes the obstacles to delivery of direct direct administration of ssDNA by lipofection, direct cellular uptake, and/or microinjection.

Another object of the present invention is to provide a method and a DNA construct for producing ssDNA *in vivo* that contains a DNA sequence with catalytic activity with the DNA enzyme inserted between flanking 5' and 3' sequences that are complimentary to an mRNA sequence of interest.

Yet another object of the present invention is to provide all the enzymatic functions that are necessary to carry out the production of ssDNA *in vivo* containing a sequence with enzymatic activity against a target mRNA of choice within a single or dual plasmid delivery system.

Another object of the present invention is to provide a method and constructs that demonstrate removal of unwanted vector sequences from *in vivo* and/or *in vitro* produced ssDNA including sequences enzymatic activity by premature termination of a cDNA product by reverse transcriptase activities and predefined termination of the cDNA transcript by secondary structure formation of the template mRNA.

Another object of the present invention is to provide a method and construct using any one of a multitude of enhancer/promoter sequences to guide production of mRNA and mRNA template for reverse transcription into a single-stranded cDNA product containing a "DNA enzyme" *in vivo*.

Another object of the present invention is to provide a single plasmid based system in which the primer binding site (i.e., the start of reverse transcription and of *in vivo* synthesis of single-stranded cDNA) is located in the 3' untranslated region of the mRNA coding for the reverse transcriptase (RNA-dependent DNA polymerase).

Another object of the present invention is to provide a single plasmid based system which proceeds with ssDNA production when transfected into eukaryotic cells which relies on endogenously produced reverse transcriptase (e.g., in the case of cells infected with HIV or primate SIV) through mRNA template recognition by compatible recognition of the PBS by the endogenous reverse transcriptase.

Another object of the present invention is to provide a method, and pharmacologically acceptable compositions, for delivering an inhibitory nucleic acid sequence including a sequence with enzymatic activity to target cells in a manner which produces a therapeutic effect.

This listing of the objects of the present invention is not intended to be a list of all of the objects of the invention. There are a vast number of other cellular functions which are mediated by the cellular genome which, in the interest of brevity and practicality, are not mentioned here and which are amenable to regulation by *in vivo* production of ssDNA. For instance, exonucleases digest ssDNA much more aggressively than double-stranded DNA (dsDNA). Consequently, another object of the present invention is to provide a ssDNA construct, and a method of producing that construct *in vivo*, which is not as susceptible to degradation by native exonucleases in the cell as ssDNA. It can be seen from this illustration that this list of some of the objects of the present invention is provided for purposes of exemplification and is not intended to limit the scope of the present invention.

SUMMARY OF THE INVENTION

These objects, and the many others which will be made apparent to those skilled in the art by the following description of the presently preferred embodiments of the invention, are achieved by providing a set of genetic elements for delivery to a cell in a vector comprising a cassette comprised of a sequence of interest flanked by an inverted tandem repeat, the cassette also comprising a sequence having enzymatic activity, and a primer binding site located 3' to the inverted tandem repeat. The set of genetic elements may also include a second sequence of interest 3' to the inverted repeats for premature termination of transcription reactions which may also include a sequence having enzymatic activity, a reverse transcriptase/RNAse H gene, and either constitutive or inducible eukaryotic promoter(s)/enhancer(s) for the RNA-dependent DNA polymerase. The set of

genetic elements may also include a restriction endonuclease gene and an appropriate promoter for the particular restriction endonuclease being utilized. In a particularly preferred embodiment, the vector into which the set of genetic elements is incorporated is a plasmid and that plasmid is transfected into a suitable host cell.

In another aspect, the present invention comprises a method for producing a single-stranded inhibitory nucleic acid which is an aptamer in a host cell. In another embodiment, the inhibitory nucleic acid produced by this method is an antisense sequence. In another embodiment, the inhibitory nucleic acid is a deoxynucleic acid squence which ihibits mRNA production by cleaving or digesting a target mRNA with a sequence with enzymatic activity against mRNA. In other embodiments, the inhibitory nucleic acid produced by this method is used to deliver a triplex-forming sequence or a sequence that is recognized and bound by a specific DNA-binding protein, or other nucleic acid and/or protein, that functions in cellular metabolism and/or replication.

The method comprises the encoding of the oligonucleotide into a complementary sequence of interest which is included in a cassette which includes a sequence of interest, a sequence having enzymatic activity (when in single stranded form), and an inverted tandem repeat. A gene encoding an RNA-dependent DNA polymerase, which preferably includes an RNase H gene and an inducible or constitutive eukaryotic promoter/enhancer appropriate for that polymerase/RNase H gene, is delivered to a host cell along with the cassette. A gene encoding a restriction endonuclease (RE) and, in the preferred embodiment, an appropriate promoter/enhancer for that RE gene, may also be delivered to the host cell. The cassette (including the sequence of interest, enzymatic sequence, and the inverted tandem repeat) and the RNA-dependent DNA polymerase/RNAse H gene are transcribed by the cell under control of their respective promoter(s)/enhancer(s). The normal function of the target cell causes the resulting mRNA transcript of the polymerase and RE genes to be translated, providing all that is needed for production of ss-DNA from the mRNA transcript of the sequence of interest. Specifically, the RNA-dependent DNA polymerase converts the mRNA transcript of the sequence of interest, sequence having enzymatic activity (when single stranded), inverted tandem repeat, and PBS to ss-cDNA, the ss-cDNA forms a stem-loop intermediate as the nucleotide bases comprising the inverted tandem repeats pair up by Watson-Crick base pairing, and the restriction

endonuclease produced from the RE gene digests the double-stranded portion of the stem-loop intermediate to "free" the single stranded DNA oligonucleotide from the loop portion of the stem-loop intermediate. After digestion of the stem, the ssDNA sequence of interest is able to fold or otherwise form the secondary structure which confers enzymatic activity of the DNA enzyme included in the sequence of interest.

Also provided are methods and plasmids for producing stem-loop intermediates *in vivo* which are capable of variable amounts of read through of a stem-loop mRNA intermediate or early termination as the ss-cDNA is being synthesized for regulating production of the ss-cDNA from one or more sequences of interest. In other words, the present invention provides methods for production and regulation of in vivo ss-cDNA by secondary folding of mRNA-directed ss-cDNA transcripts.

Multiple systems can be used to deliver the cassette to the target cell to direct the synthesis of ssDNA within the cell, including plasmid or plasmid-based vector systems or viral based vector systems, and these systems are adapted for that purpose in accordance with standard delivery techniques currently known to the skilled practitioner. These systems include, but are not limited to, viral based systems such as adenovirus, adenoassociated virus, retroviral vectors, and conjugate vectors using double stranded plasmid DNA based transfection systems. All such systems are contemplated by the present invention. Once inside the cell, the set of genetic elements including the cassette is transcribed in the normal course of cell metabolism, producing an mRNA transcript of the sequence of interest that is then converted to cDNA by the reverse transcriptase which is likewise produced by the cell from the reverse transcriptase/RNase H gene included in the cassette under the control of the promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of an mRNA transcript setting out the arrangement of the various components of the set of genetic elements which are expressed in the cell in accordance with the method of the present invention.

Figure 2 is a schematic representation of the binding of an antisense sequence produced in accordance with the present invention which incorporates the "10-23 DNA enzyme" binding to a target mRNA and the subsequent cleavage of the target mRNA. Figure 2A is an enlarged representation of the binding of the antisense sequence of Fig. 2

to the target mRNA showing the interaction of the 10-23 DNA enzyme (included in a generalized inhibitory nucleic acid sequence) with the cleavage site of the target mRNA.

Figure 3A is a schematic map of the plasmid pssDNA-Express A constructed in accordance with the present invention. Figure 3B is a schematic map of the plasmid pssDNA-Express-A after deletion of the Mbo II gene by digesting with Xma I and Sac II.

Figure 4A is a schematic map of the starting plasmid pcDNA3.1Zeo$^+$ (Invitrogen, Inc.) for plasmid pcDNA3.1Zeo$^+$/NM-link2-gag/pss-DNA-Express-4B constructed in accordance with the present invention. Figure 4B is a schematic map of pcDNA3.1Zeo$^+$/NM-link2-gag (also designated pssDNA-Express-4B). Figure 4C is a schematic representation of the cloning sites, inverted repeats, and PBS region (arrow) for pcDNA3.1Zeo$^+$/NM-link2-gag/pssDNA-Express-4B. Figure 4D is a sequence map of the cloning sites, inverted repeats, and PBS region (arrow) for pcDNA3.1Zeo$^+$/NM-link2-gag/pssDNA-Express-4B.

Figure 5A is a schematic map of the plasmid pssDNA-Express-C constructed in accordance with the present invention. Figure 5B is a schematic representation of the cloning sites, inverted repeats, and PBS region (arrow) for plasmid pssDNA-Express-C. Figure 5C is a sequence map of the cloning sites, inverted repeats, and PBS region (arrow) for plasmid pssDNA-Express-C.

Figure 6A shows the partial sequence of the 23rd codon of h-ras antisense binding sequence with the 10-23 DNA enzyme sequence inserted between the 5' and 3' complimentary sequences. Figure 6B shows the partial sequence of c-raf kinase antisense binding sequence with the 10-23 DNA enzyme sequence inserted between the 5' and 3' complimentary sequences. Figure 6C shows the partial sequence of pleiotrophin antisense binding sequence with the 10-23 DNA enzyme sequence inserted between the 5' and 3' complimentary sequences. Figure 6D shows the partial sequence of tat antisense binding region of the SIV sequence with the 10-23 DNA enzyme sequence inserted between the 5' and 3' complimentary sequences.

Figure 7 shows a Northern blot of an antisense producing vector constructed in accordance with the present invention producing an antisense sequence against c-raf kinase (Fig. 6B) and including the "10-23 DNA enzyme" after transfection of plasmid pssDNA-Express-C (Fig. 5) including that sequence into a lung cancer cell line *in vitro*.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In this description of the preferred embodiments of the present invention, methods and nucleic acid constructs are described for use in producing single-stranded deoxyribonucleic acid (ss-cDNA) oligonucleotides of virtually any predefined or desired nucleotide base composition *in vivo* in eukaryotic cells with or without flanking nucleotide sequences. Methods and constructs are described which use biological rather than the *in vitro*, or artificial chemical synthesis of ss-cDNA of desired nucleotide base composition. Because biological, i.e., enzymatic reactions, are used in these methods they are applicable to any *in vivo* system.

A vector (as used herein, the term "vector" refers to a plasmid or modified viral construct used to deliver and manipulate DNA segments of interest) system was designed to produce any DNA sequence as a ss-cDNA molecule, free of most contiguous vector sequence, within mammalian cells. The vector system contains all the necessary enzymatic functions and signaling instructions to allow the host cell to produce ss-cDNA. The cell to which the vector of the present invention is delivered produces an RNA transcript (Fig. 1), driven by an eukaryotic promoter just as the above-described enzymes are driven by eukaryotic promoters, which is used as a template to direct the synthesis of any desired single-stranded DNA sequence (a "sequence of interest"). In more detail, a description is set out herein of a system in which the vector comprises two plasmids that are co-transfected into a suitable host cell, which can be any eukaryotic cell, to produce ssDNA having the sequence of interest in the cell. A second system is described in which the vector system comprises a single plasmid containing the sequence of interest that is transfected into a suitable host cell for production of an inhibitory nucleic acid, such as the antisense sequence shown in Fig. 1, from the sequence of interest.

Inhibitory nucleic acids may be ssDNA synthesized from the mRNA template or the mRNA template itself, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA--RNA, a DNA--DNA, or RNA-DNA duplex or triplex is formed. More commonly, these nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene, but the "sense" sequence is also utilized in the cell for therapeutic purposes. For example, the identification of oligonucleotides that specifically bind to biomolecules

that do not normally bind to RNA or DNA has now been demonstrated for a number of biomolecules that vary widely in size, structure and composition. Examples of such molecules include (but are not limited to): (1) thrombin, a multifunctional regulatory protein that converts fibrinogen to fibrin in the process of clot formation; (2) bradykinin, a nonapeptide kinin involved in blood pressure regulation and implicated in hypotension; (3) PGF2 alpha, a prostaglandin or fatty acid derivative that exhibits hormonal activity. Additionally, the interaction of oligonucleotides with biomolecules whose natural biological function is primarily extracellular has now been demonstrated (*see*, for instance, U.S. Patent No. 5,840,867). The term "inhibitory nucleic acids" as used herein, therefore, refers to both "sense" and "antisense" nucleic acids.

By binding to the target nucleic acid, an inhibitory nucleic acid inhibits the function of the target nucleic acid. This inhibitory effect results from, for example, blocking DNA transcription, processing or poly(A) addition to mRNA, DNA replication, translation, or promoting inhibitory mechanisms of the cells (such as promoting RNA degradation). Inhibitory nucleic acid methods therefore encompass a number of different approaches to altering expression of genes. These different types of inhibitory nucleic acid technologies are described in Helene, C. and J. Toulme, 1049 Biochim. Biophys. Acta. 99-125 (1990), which is referred to hereinafter as "Helene and Toulme," and which is incorporated herein in its entirety by this specific reference thereto.

In brief, inhibitory nucleic acid therapy approaches can be classified into (1) those that target DNA sequences, (2) those that target RNA sequences (including pre-mRNA and mRNA), (3) those that target proteins (sense strand approaches), and (4) those that cause cleavage or chemical modification of the target nucleic acids such as the ssDNA enzymes, including the so-called "10-23 enzyme" as described herein. The first approach contemplates several categories. Nucleic acids are designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. More commonly, inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are used to prevent maturation of pre-mRNA. Inhibitory nucleic acids may be designed to interfere with RNA processing, splicing or translation. In the second

approach, the inhibitory nucleic acids are targeted to mRNA. In this approach, the inhibitory nucleic acids are designed to specifically block translation of the encoded protein. Using this second approach, the inhibitory nucleic acid is used to selectively suppress certain cellular functions by inhibition of translation of mRNA encoding critical proteins. As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms to inhibit translation of the encoded protein(s). An example of such an inhibitory nucleic acid is the sequence that is complementary to regions of c-myc mRNA, which inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which overexpresses the c-myc proto-oncogene (Wickstrom E. L., *et al.*, 85 Proc. Natl. Acad. Sci. USA 1028-1032 (1988) and Harel-Bellan, A., *et al.*, 168 Exp. Med. 2309-2318 (1988)).

The inhibitory nucleic acids produced in the cell can also utilize the third approach of designing the "sense" strand of the gene or mRNA to trap or compete for enzymes or binding proteins involved in mRNA translation as described in Helene and Toulme. Lastly, inhibitory nucleic acids are used to induce chemical inactivation or cleavage of the target genes or mRNA. Chemical inactivation occurs, for instance, by induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell and by the method contemplated herein, namely, the cleavage of the target mRNA by the sequence having enzymatic activity against mRNA that is incorporated into the cassette of the present invention.

In brief, in a first aspect, the present invention comprises a set of genetic elements adapted for delivery into a cell to produce ss-DNA *in vivo*. The set of genetic elements is incorporated into at least one vector for delivery into the cell and, as shown in Fig. 1, includes a RNA dependent DNA polymerase (reverse transcriptase) gene, a sequence of interest located either between inverted repeats (IR) or 3' to the inverted repeats, a primer binding site (PBS) for the reverse transcriptase that is located 3' to the cassette (the cassette including the sequence of interest and the inverted repeats), a sequence having enzymatic activity (when able to fold into the secondary structure which confers enzymatic activity upon the sequence) located inside the sequence of interest (whether the sequence of interest is located between inverted repeats such that it is also part of the cassette or 3' to the inverted repeats), and the functions and signaling instructions for transcription of

these components *in vivo*. The vector system also preferably includes the functions and signaling instructions for translation of the reverse transcriptase (RT) gene. An additional component is a restriction endonuclease (RE) gene, which may be included for a purpose described below.

In the vector system described herein, the vector comprises a two plasmid system, and the set of genetic elements that are adapted for delivery to the cell to produce ssDNA *in vivo* include the RNA-dependent DNA polymerase (reverse transcriptase) gene, which additionally contains an RNAse H gene, linked with a histidine-proline linker to a restriction endonuclease gene. These genes were constructed and inserted into a plasmid vector which contains the necessary transcriptional and translational control elements along with polyadenylation tailing sequences. This plasmid is referred to herein as the "A" plasmid (pssDNA-Express-A as shown in Fig. 3 in one of the preferred embodiments described herein). A second, "B" plasmid was constructed which, in the embodiment described herein, includes four of the above-listed elements, namely, a primer binding sequence (PBS) matched to the reverse transcriptase, a sequence having enzymatic activity (when released as ssDNA), a sequence of interest, and an inverted tandem repeat. In the embodiment described herein (pssDNA-Express-4B as shown in Fig. 4), the "DNA enzyme" is included in one of the plasmids as part of the sequence of interest and is positioned either between the inverted tandem repeats or in a 5' position (with respect to the mRNA transcript) to the primer binding sequence (PBS), the PBS being located at the most 3' aspect of the mRNA transcript. In other words, the sequence of interest including the DNA enzyme is located (1) inbetween the inverted repeats, (2) between the inverted repeats and the PBS, and/or (3) both between the inverted repeats and inbetween the inverted repeats and the PBS. Like plasmid A, plasmid B also includes a combination of transcriptional control elements. In the preferred embodiment described, however, plasmid B does not include (or require) translational control elements since no protein product is produced from this construct.

In the single plasmid vector system described herein, all of the elements of the set of genetic elements listed above were included in a single "C" plasmid with the exception of the RE gene, which was not included for reasons that will be made clear from the following discussion. Additionally, the components included in plasmid B of the two

plasmid system described above, e.g., the primer binding sequence (PBS), DNA enzyme sequence, sequence of interest, and inverted tandem repeat, reside in the untranslated 3' portion of the reverse transcriptase polyprotein in the C plasmid. In other words, when the RT-RNAse H component of the C plasmid is transcribed under control of an appropriate promoter (in the preferred embodiments described herein, the RSV promoter was utilized), the resulting mRNA transcript contains the coding region for the reverse transcriptase-RNAse H polyprotein and, at the end of translation at the stop signals, the additional mRNA transcript contains (3' to this translated protein) the four elements from the B plasmid with further 3' downstream signaling events for polyadenylation signals, which remain intact from the RT-RNAse H component. The single plasmid vector system described herein (pssDNA-Express-C as shown in Fig. 5) does not contain the restriction endonuclease gene, and therefore does not include the ability to digest the stem of the stem-loop intermediate formed by the inverted repeats. Consequently, the sequence of interest (including the DNA enzyme) is inserted into the C plasmid only in a 3' position to the inverted repeats and unwanted vector sequences are removed by premature truncation of the ss-cDNA product as the transcript encounters the relatively stable stem and is unable to continue transcribing the ss-cDNA from the mRNA transcript. More specifically, as will be made apparent in the following description, each of the sequences of interest (including the DNA enzyme sequences) was inserted only within the Bam H I-Pac I restriction sites of the pssDNA-Express-C plasmid.

As will also be apparent from the following description of the B and C plasmids, the plasmids include cloning sites for insertion of the sequence of interest. Both Not 1 sites (located between the inverted repeats) and Pac I/BamH I (3' to the inverted repeats, e.g., between the inverted repeats and the PBS) sites are provided in the preferred embodiment of the B plasmid described herein. The preferred C plasmid described herein includes only the Pac I/BamH I sites for this purpose. However, those skilled in the art who have the benefit of this disclosure will recognize that these particular cloning sites were chosen for the particular systems described herein and that other cloning sites may be equally useful for this same purpose. The particular A plasmid described herein was not intended to include the sequence of interest, but those skilled in the art having the benefit of this disclosure will recognize that, if a two plasmid vector system is to be used, the

elements of the set of genetic elements of the present invention, and particularly the sequence of interest, may be inserted into either plasmid as may be convenient.

The nucleic acid sequence that is referred to herein as a cassette provides the template for synthesis of ss-cDNA in target cells. It is this element which includes the sequence of interest, inverted tandem repeats, and primer binding site. As is the case for the other elements of the set of genetic elements of the present invention, this genetic element is preferably regulated by an appropriate wide spectrum or tissue-specific promoter/enhancer, such as the CMV promoter, or combination of promoters/enhancers, located upstream of the genetic element. Also as is the case for the other genetic elements, the promoter/enhancer can either be constitutive or inducible promoter. Those skilled in the art who have the benefit of this disclosure will recognize that a number of other eukaryotic promoters may be used to advantage to control expression of the sequence of interest including SV-40, RSV (non-cell type specific) or tissue specific glial fibulary acidic protein (GFAP).

As shown in Figs. 1 and 2, for expression in eukaryotic cells, the cassette also includes a downstream polyadenylation signal sequence so that the mRNA produced by the sequence of interest has a poly(A) tail. Between the 3' inverted tandem repeat and the polyadenylation signal, the genetic element contains a primer-binding site (PBS) for initiation of priming for cDNA synthesis. The PBS is a sequence which is complementary to a transfer RNA (tRNA) which is resident within the eukaryotic target cell. In the case of the mouse Maloney reverse transcriptase described herein as being utilized in conjunction with the present invention, the PBS takes advantage of the proline tRNA. The PBS utilized in connection with the presently preferred embodiment of the invention that is described herein was taken from the actual 18 nucleotide sequence region of mouse Moloney virus. *See* 293 Nature 81. In the case of the reverse transcriptase gene from human immunodeficiency virus that was also tested as noted above, the PBS used was taken from the nucleotide sequence of HIV. Y. Li, *et al.*, 66 J. Virology 6587-6600 (1992). In short, any PBS that is matched to the reverse transcriptase which is utilized in connection with the method of the present invention is utilized for this purpose.

The sequence of interest element comprising the cassette is any sequence of interest including a DNA sequence with catalytic activity against mRNA. However,

because of the inclusion of the DNA enzyme in the sequence of interest, the present invention particularly contemplates that the sequence of interest is an inhibitory nucleic acid that is an antisense sequence. For that reason, the following examples describe production of four antisense sequences of interest as set out in Fig. 6, each including a sequence having enzymatic activity against mRNA, including C-raf kinase, h-ras antisense sequence, antisense sequence to pleiotrophin angiogenic growth factor, and the tat region of simian immunodeficiency virus (SIV).

The nucleic acid sequence having enzymatic activity which comprises the cassette of the present invention can be any of several such known sequences. The sequence having the desired catalytic activity is inserted into the cassette in either or both of the two locations, e.g., between the inverted repeats and inside the sequence of interest or inside the sequence of interest that is located 3' to the inverted repeats and 5' to the PBS (see Fig. 1). Either way, the resulting aptamer is specific for the target of the sequence of interest and is therefore used to target mRNA, inhibit or change mRNA splicing mechanisms, or even directly alter the cellular genome in a specific manner. The catalytic ssDNA aptamers are also combined with each other to perform independent, but coordinated, functions in the cell. For instance, a sequence with DNA cleaving ability is combined with a second sequence with DNA ligase activity to splice and repair specific mutations in the cellular genome. Similarly, such a combination of digestive and repair enzymes is used to introduce site-specific mutagenic sequence changes in the cellular genome.

Nucleic acid sequences with enzymatic activity that are appropriate for use in connection with the present invention include, but are not limited to,

sequences having RNAse activity such as the so-called "10-23" and "8-17 enzymes" (Santoro, S.W. and G.F. Joyce, *supra* (1997)) and other metal-dependent RNAses (Breaker, R.R. and G.F. Joyce, 1 Biol. Chem. 223-229 (1994) and Breaker, R.R. and G.F. Joyce, 2 Biol. Chem. 655-660 (1995)) and histidine-dependent RNAse (Roth, A. and R.R. Breaker, 95 Proc. Natl Acad. Sci. USA 6027-6031 (1998);

sequences having DNAse activity such as copper-dependent DNAse (Carmi, N., *et* al., 3 Chem. Biol. 1039-1046 (1996), Carmi, *et al.*, *supra* (1997);

Sen, D. and C.R. Geyer, 2 Curr. Opin. Chem. Biol. 680-687 (1998)) and the DNAses which required divalent metal ions as cofactors or hydrolyzed the substrate independently of divalent metal ions reported in Faulhammer, D. and M. Famulok, *supra*;

sequences with DNA ligase activity such as copper-dependent DNAse (Breaker, R.R., 97 Chem. Rev. 371-390 (1997) and zinc-dependent E47 ligase (Cuenoud, B. and J.W. Szostak, *supra*);

sequences with DNA kinase acitivity such as calcium-dependent DNA kinase (Li, Y., *supra*); and

sequences with RNA kinase acitivity such as calcium-dependent DNA kinase (Li, Y., *supra*).

The particular nucleic acid sequence having enzymatic activity utilized in the examples described herein is the "10-23 enzyme" (Santoro, S.W. and G.F. Joyce, *supra* (1997)), and it is this enzymatic sequence which is represented schematically in Figs. 2 and 2A. However, those skilled in the art will recognize from this disclosure that any of the sequences reported in the above-listed literature will function for the intended purpose when inserted into the cassette of the present invention.

In the presently preferred embodiment, the sequences of interest are delivered to a host cell either by co-transfection of the cells with two plasmids, designated pssDNA-Express-A and pssDNA-Express-4B, each plasmid being designed and constructed to include the components listed above, or the single pssDNA-Express-C plasmid. To summarize, the plasmid (pssDNA-Express-4B in the preferred embodiment) encodes the sequence of interest, nested within flanking sequences that include the inverted repeats, and the primary binding site that provides the post-transcriptional processing signals that mediate the conversion of the mRNA into single-stranded DNA. The B plasmid also includes the second sequence of interest when this second aspect of the invention is utilized and as set out above, when the RE gene is omitted from the construct of the present invention, for instance in the single "C" plasmid described herein, it is this second sequence of interest that encodes the inhibitory nucleic acid having the desired activity. Activities required for processing the primary gene product of the B plasmid into single-stranded DNA, with the removal of vector sequences and processing signals, specifically

the reverse transcriptase/RNAse H, and restriction endonuclease, are expressed from the A plasmid (pssDNA-Express-A in the preferred embodiment). The single-stranded DNA sequence that is released by interaction of the transcriptional products of these components *in vivo* is free to bind intracellular targets such as mRNA species and DNA promoters in antisense and triplex strategies.

As noted above, as described herein, the B plasmid pssDNA-Express-B includes cloning sites (*Not* I sites were utilized in the preferred embodiment of the B plasmid described herein) between which any DNA sequence of interest is placed (as noted above, in the examples described herein, the sequences include h-ras, c-raf kinase, a region encoding the angiogenic growth factor pleiotrophin, and the region encoding tat from SIV). Flanking the cloning sites are signals directing the processing of the primary mRNA transcript, produced from a promoter (a CMV promoter was utilized in the preferred B plasmid described herein), into the desired single-stranded inhibitory nucleic acid. After cloning of the desired sequence of interest into the B plasmid, the A and B plasmids are co-transfected into a cell line of choice for constitutive expression of ssDNA. Similarly, in the single ("C") plasmid system described herein, the sequence of interest is cloned into that plasmid and transfected into the cell line for further processing. Regardless of the distribution of the elements of the above-described set of genetic elements between two (or even more) plasmids, or if the elements are all contained in a single plasmid, this processing proceeds in three steps following transcription of the single-stranded DNA region (i.e., sequence of interest, inverted repeats, and PBS):

(1) reverse transcription of the B or C plasmid RNA transcript by a reverse transcriptase, which in the preferred embodiment described herein is reverse transcriptase expressed by the A or C plasmid (in the preferred embodiment described herein, the reverse transcriptase is Moloney mouse leukemia virus (MoMuLV) reverse transcriptase), proceeding from the primer binding site lying 3' to the sequence of interest (including the sequence with enzymatic activity), inverted repeats, and primer binding site as shown in Fig. 1;

(2) RNAse H digestion of the resulting heteroduplex, either by the RNAse H activity of the reverse transcriptase polyprotein or by endogenous

RNAse H activity, to release the single-stranded DNA precursor from its RNA complement; and

(3) Premature termination of the cDNA transcript by formation of the stem-loop secondary structure by the self-complementary inverted tandem repeats.

Those skilled in the art will recognize from this disclosure that the particular cloning sites flanking the sequence of interest, the particular reverse transcriptase, restriction endonuclease (if utilized), promoter, primer binding site, and all the other elements of the set of genetic elements of the present invention are chosen depending upon the particular sequence of interest and/or system in which the ssDNA is to be expressed.

Regarding the RNA-dependent DNA polymerase, or reverse transcriptase (RT) gene which is the first component of the present invention, as noted above, the reverse transcriptase/RNAse H gene from Moloney murine leukemia virus was used to advantage in the examples described herein. The reverse transcriptase/RNAse H gene from the human immunodeficiency virus (HIV) has also been tested. Many other retroviral reverse transcriptase/RNAse H genes may be used to advantage in connection with the present invention, it being preferred that the reverse transcriptase/RNase H gene be a reverse transcriptase/RNase H gene that is regulated by an appropriate upstream eukaryotic promoter/enhancer such as the CMV or RSV promoter for expression in human cells.

Many RNA-dependent DNA polymerase/reverse transcriptase genes are known which are suitable for use in connection with the present invention including those from retroviruses, strains of hepatitis B, hepatitis C, bacterial retron elements, and retrons isolated from various yeast and bacterial species. As found in nature, these RNA-dependent DNA polymerases usually have an associated RNase H component enzyme within the same coding transcript. However, the present invention does not require the naturally-occurring RNase H gene for a particular reverse transcriptase. In other words, those skilled in the art will recognize from this disclosure that various combinations of reverse transcriptase and RNase H genes can be spliced together for use in connection with the present invention to fulfill this function and that modifications and/or hybrid versions of these two enzyme systems are available and/or known to those skilled in the art which will function in the intended manner. Those skilled in the art will also recognize that the target cell may itself have sufficient endogenous RNase H to fulfill this function.

Similarly, those skilled in the art will recognize that the target cell may itself have sufficient endogenous reverse transcriptase activity from, for instance, prior retroviral infection, to fulfill this function.

Those skilled in the art who have the benefit of this disclosure will also recognize that a number of tissue-specific or wide spectrum promoters/enhancers, or combinations of promoters/enhancers, other than those described herein may also be used to advantage to control the reverse transcriptase/RNAse H gene, the RE gene, and the sequence of interest. Although a list of all available promoters/enhancers is not needed to exemplify the invention, as noted above, the promoters/enhancers may be constitutive or inducible and may include the CMV or RSV promoters/enhancers listed here and many other viral or mammalian promoters. Representative promoters/enhancers which are appropriate for use in connection with the present invention may include, but are not limited to, HSVtk (S.L. McKnight, *et al.*, 217 Science 316 (1982)), human ß-globulin promoter (R. Breathnach, *et al.*, 50 Ann. Rev. of Biochem. 349 (1981)), ß-actin (T. Kawamoto, *et al.*, 8 Mol. Cell Biol. 267 (1988)), rat growth hormone (P.R. Larsen, *et al.*, 83 Proc. Natl. Acad. Sci. U.S.A. 8283 (1986)), MMTV (A.L. Huang, *et al.*, 27 Cell 245 (1981)), adenovirus 5 E2 (M.J. Imperiale, *et al.*, 4 Mol. Cell. Biol. 875 (1984)), SV40 (P. Angel, *et al.*, 49 Cell 729 (1987)), α-2-macroglobulin (D. Kunz, *et al.*, 17 Nucl. Acids Res. 1121 (1989)), MHC class I gene H-2kb (M.A. Blanar, *et al.*, 8 EMBO J. 1139 (1989)), and thyroid stimulating hormone (V.K. Chatterjee, *et al.*, 86 Proc. Natl. Acad. Sci. U.S.A. 9114 (1989)).

The reverse transcriptase/RNase H gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA produced from the reverse transcriptase/RNase H gene includes a 3' poly(A) tail for mRNA stability. As known to those skilled in the art, multiple poly(A) tails are available and are routinely used for production of expressed eukaryotic genes.

The reverse transcriptase produced in the cell synthesizes a complementary DNA (cDNA) using as the template the genetic element including the sequence of interest described below. The RNase H activity of the reverse transcriptase degrades the mRNA template component of the RNA/cDNA hybrid to produce ss-DNA *in vivo*.

The gene encoding the restriction endonuclease (used only in the two plasmid system, and not even a required component of that system) may be any of several genes which encode for restriction endonucleases, and preferably those that are controlled by one or more constitutive or inducible wide spectrum and/or tissue-specific promoters/enhancers such as those listed above. The particular restriction endonucleases tested were MboII and FokI, Mbo II being preferred, but those skilled in the art who have the benefit of this disclosure that any restriction endonuclease (type I, II, IIS, or III) site may be included in the inverted tandem repeat. These enzymes "clip" or digest the stem of the stem-loop intermediate described below to linearize the sequence of interest as single-stranded DNA.

Although expression of the RE gene may be regulated by an appropriate constitutive or inducible promoter/enhancer located upstream from the restriction endonuclease gene such as the CMV or RSV promoter for expression in human cells, in the preferred plasmid pssDNA-Express-A, the RE gene (MboII) is linked to the RT-RNAse H polypeptide. Those skilled in the art who have the benefit of this disclosure will also recognize that several other promoters/enhancers such as those listed above may be used to advantage to control the RE gene just as various promoters/enhancers are available for the reverse transcriptase/RNase H gene as discussed above. The RE gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA transcript from the restriction endonuclease gene will have a 3' poly(A) tail.

In the second aspect of the invention, a second sequence of interest which includes the nucleotide sequence for one or more DNA enzymes is inserted between the primer binding site and the 3' inverted tandem repeat (see Fig. 1). The second sequence of interest is likewise reverse transcribed from the PBS. Depending upon the stabilility of the stem loop secondary structure, reverse transcription is terminated at the stem loop formed in the mRNA transcript of the cassette such that only the second sequence of interest is produced as ssDNA in the host cell. Alternatively, the second sequence of interest is reverse transcribed along with the inverted repeat and the first sequence of interest. In a third aspect of the invention, either of the first or second sequences of interest are included in the cassette with multiple additional sequences of interest, each with its corresponding promoter/enhancer, polyadenylation signal, and PBS (inside or outside of the inverted

tandem repeats), all of which are utilized for producing ssDNA *in vivo* for, for instance, delivering an inhibitory nucleic acid to the target cell.

The inverted tandem repeats cause the ss-cDNA to fold back upon itself to form the stem of a stem-loop structure in the manner described in my co-pending application Serial No. 08/877,251, the specification of which is hereby incorporated into this application in its entirety by this specific reference. The folding of the ss-cDNA in this fashion occurs after the sequence of interest and its flanking inverted repeats are transcribed in the cell and after the reverse transcriptase/RNase H (produced by transcription and translation of RT/RNAse H genes which also comprise elements of the set of genetic elements of the present invention) produce the ss-cDNA sequence of interest from the mRNA transcript in the cell. In the case of those plasmids which contain an RE gene, the stem comprises one or more restriction endonuclease site(s) which is cut by the restriction endonuclease produced from the restriction endonuclease gene likewise coded for by the RE gene as described in that same co-pending application Serial No. 08/877,251. The ss-cDNA which is produced is transcribed with the encoded 5' and 3' regions flanking the stem (made up of the inverted repeats) and the loop (containing the sequence of interest). This stem structure is comprised of double stranded, anti-parallel DNA and is designed to contain one or more restriction endonuclease recognition sites within the double stranded portion, i.e., the inverted repeats. In this manner, the stem is cut (also termed digested or cleaved) by any of the many restriction endonuclease enzymes which recognize the cut site designed into the stem (note that the endonuclease recognition site may be designed into the stem even though the RE gene is not included in the vector system of the present invention). The loop portion of the ss-cDNA, which does not form any apparent duplex DNA, is immune to restriction endonuclease activity since restriction endonucleases recognize only double stranded DNA as a target substrate.

It will be recognized by those skilled in the art that the restriction endonuclease site(s) need not be designed into the inverted repeats which form the stem of the stem-loop intermediate if the second aspect of the present invention is being utilized. In other words, if it is desired to produce ssDNA from a second sequence of interest located between the primer binding site and the inverted repeats, with transcription of the cassette to terminate at the stem formed by the inverted repeats, there is no need for a restriction

endonuclease site in the stem. Another option is to design the inverted repeats to contain eukaryotic, prokaryotic, or viral protein DNA binding sites, which can act to competitively titer out selected cellular proteins. Combinations of restriction sites or other sequence specific elements may be included in the inverted tandem repeats depending on the base pair composition chosen for the construction of inverted repeats such that linear or precisely cut stem-loop intermediate forms of ss-DNA are produced. It is generally preferred to use synthetically constructed sequence specific elements in the inverted tandem repeats since it is unlikely that a naturally occurring inverted repeat would have the properly aligned restriction sites.

When the elements comprising the set of genetic elements of the present invention are incorporated into a vector, it is preferred that the vector contain other specialized genetic elements to facilitate the identification of cells that carry the vector and cassette or to increase the level of expression of the cassette. The specialized genetic elements include selectable marker genes so that the vector can be transformed and amplified in a prokaryotic systems. For example, the most commonly used selectable markers are genes that confer to the bacteria (e.g., E. *coli*) resistance to antibiotics such as ampicillin, chloramphenicol, kanamycin (neomycin), or tetracycline. It is also preferred that the vector contain specialized genetic elements for subsequent transfection, identification and expression in eukaryotic systems. For expression in eukaryotic cells, multiple selection strategies may be used that confer to the cell (e.g., Chinese Hamster Ovarian: CHO) resistance to an antibiotic or other drug or alter the phenotype of the cell such as morphological changes, loss of contact inhibition, or increased growth rate. Selectable markers used in eukaryotic systems include, but are not limited to, resistance markers for Zeocin, resistance to G418, resistance to aminoglycoside antibiotics, or phenotypic selection markers such *β-gal* or green fluorescence protein.

Incorporation of these components into an appropriate vector allows two convenient methods for removing predetermined vector sequences after the production of ssDNA. In the first method, the loop portion of the ssDNA stem-loop intermediate that is produced is comprised of the nucleotide sequence of interest and, after digestion with the restriction endonuclease, the loop is released as linearized, single-stranded cDNA without

any flanking sequences. In the second method in which the cassette is reverse transcribed from the PBS and a second sequence of interest is included in the cassette 3' to the inverted tandem repeat, reverse transcription is terminated at the stem of the stem-loop structure such that the resulting ssDNA is produced without flanking sequences. If it is desired to produce ssDNA utilizing the second method, the cassette is designed with inverted repeats which form a stem that is more stable than the stem needs to be if the ssDNA is produced by digestion of the stem in accordance with the first aspect of the present invention. By designing the cassette with inverted repeats that form a stem that is easily denatured in accordance with the first aspect of the invention, reverse transcription proceeds right on through the second sequence of interest (if it is even designed into the cassette) to the sequence of interest located between the inverted repeats. A stem which is intermediate in stability allows production of both the first and second sequences of interest.

As noted above, premature truncation of the ss-cDNA transcript in accordance with the second aspect of the present invention provides the opportunity to remove unwanted vector sequences without digestion of the stem-loop structure with the restriction endonuclease. Consequently, in single plasmid system exemplified by the plasmid pssDNA-Express-C, the Mbo II restriction endonuclease gene is not included in the plasmid. In the case of pssDNA-Express-C, the Mbo II gene was removed prior to insertion of the 3' untranslated region into the plasmid. Consequently, the single plasmid of the single plasmid system includes the aforementioned inverted tandem repeats, a sequence of interest which contains a nucleic acid sequence having enzymatic activity, and a primer binding site in the truncated, 3' untranslated region of the transcribed mRNA template of the plasmid encoding the reverse transcriptase-RNAse H polyprotein.

The sequence of interest is cloned into the plasmids which represent the preferred embodiments of the vector system of the present invention (pssDNA-Express-B and pssDNA-Express-C in the examples set out below) so that the sequence of interest is transcribed from its proimoter (the cytomegalovirus promoter in the B plasmid described below), which is located just upstream of the sequence of interest, and terminates with the BGH polyA signal located just downstream of the sequence of interest. Similarly, the RT-RNAse H polyprotein is transcribed from its promoter (the RSV promoter in the examples

described herein). This mRNA transcript, which includes the cassette (inverted repeats, sequence of interest including the nucleic acid sequence having enzymatic activity, and PBS) in the untranslatable 3' end, is transported to the cytoplasm of the cell for translation by the natural host cell machinery. The reverse transcriptase (expressed from either the A or C plasmids, pssDNA-Express-A and pss-Express-C in the examples set out below) reverse transcribes the primary transcript, using host t-RNApro as a primer, from the position of the MoMuLV reverse transcriptase promoter, producing a DNA-RNA duplex molecule. Endogenous RNAse H activity, or RNAse H produced from a gene included in the vector system of the present invention (e.g., the A or C plasmids), degrades the RNA strand, releasing a single-stranded DNA. This ssDNA sequence contains inverted, complementary repeats which form the stem of a stem-loop structure. In the preferred embodiment described herein, the stem is designed to include a restriction endonuclease site, and in the examples set out below, the site is an Mbo II recognition site (GAAGA). The restriction endonuclease, if expressed from the vector system of the present invention (the A plasmid, pssDNA-Express-A in the examples described herein), cuts the double-stranded stem outside of its recognition sequence, releasing the loop region of the stem-loop intermediate as a single-stranded DNA of sequence and length defined by the cut sites and the particular sequence of interest. Note that in the embodiment described herein, the reverse transcriptase and Mbo II activities are expressed in a single protein chain encoded by a transcript produced from the RSV promoter of the A plasmid and separated by a short linker region rich in proline.

As explained in my co-pending application Serial No. 09/397,782, premature termination of the reverse transcriptase cDNA transcript at the 3' aspect of the stem structure was discovered when a vector with a stable 29 base pair stem structure was used in *in vitro* experiments, allows a unique opportunity for limiting the intervening vector sequences contained with an *in vivo*-produced ss-cDNA for use as an inhibitory nucleic acid. Specifically, this discovery makes possible a new construct having a nucleic acid sequence with enzymatic activity against mRNA located in the sequence of interest, the sequence of interest including the DNA enzyme being located 3' in relation to the stem-loop structure and 5' in relation to the PBS for production of ssDNA *in vivo*, which binds to the target mRNA and cleaves the target.

Premature reaction termination by secondary structure of the mRNA transcript is used as an alternate control mechanism to produce ssDNA sequences of various sizes *in vivo*. Limiting the extent of 3' read through of the ss-cDNA into more proximal 5' mRNA substrate by desiging the ss-cDNA to include a stable stem has the effect of reducing or eliminating the amount of unwanted 5' mRNA transcript sequences which could interfere with the desired function of the ss-cDNA product (i.e., for antisense or triplex formation). Those skilled in the art will recognize from this disclosure that the placement of such stem-loop intermediates in any desired location within the mRNA transcript allows modification of the ss-cDNA produced by reaction termination as a result of the secondary structure imposed on the transcript. It will also be apparent that a mixed population of ss-cDNA (i.e., the truncated transcript at the position between 3' PBS and 3' aspect of the inverted repeat and the read through product) is produced by transformation of host cells with the pNM-New-Link plasmid described herein and that the multiple transcripts produced may be assigned different functions in the cell. For example, if different antisense sequences are assigned to different positions in the mRNA transcript (i.e., one to the loop portion of the stem-loop secondary structure as the first sequence of interest and one to the position between the PBS and the stem as the second sequence of interest) and produced in different proportions depending upon the stability of the stem of the stem-loop structure, various levels of control are possible within the cell and at the transcriptional level due to secondary structures of the mRNA template.

As noted above, in addition to production of multiple ss-cDNA sequences *in vivo*, the present invention contemplates the purposeful introduction of cellular factors such as single and double stranded binding proteins into the host cells to recognize the double-stranded stem portion or single stranded/double stranded junction of the stem-loop transcript to add to or reduce the stability of this secondary structure. The use of these factors allows control over the amount of premature termination or read through products produced by the cell. Quantitative changes, as well as changes ss-cDNA length, are orchestrated by manipulation of the stem-loop structure within the mRNA transcript in the cell. Thus, the secondary structure of the template mRNA is utilized to produce and control the production of ssDNA *in vivo*.

It will also be evident to those skilled in the art from this description that the intact stem-loop ss-cDNA structure can function similarly in many applications as the linearized ss-cDNA form. Consequently, the cassette is also used to advantage without the restriction endonuclease gene and associated regulatory elements and/or with a sequence of interest which lacks the corresponding restriction endonuclease site.

It will also be evident to those skilled in the art from this description of the preferred embodiments of the present invention that a cassette can be made which encodes a ss-cDNA that has a "trimmed" stem-loop structure. The restriction endonuclease sites encoded in the inverted repeats flanking the sequence of interest are designed such that the stem portion (after duplex formation) is digested with the corresponding restriction endonuclease so as to cut the dsDNA comprising the stem in a way that removes a portion of the stem and the associated flanking sequences yet leaves sufficient duplex DNA that the transcript retains the above-described stem-loop structure. Such a ss-cDNA structure may be more resistant to intracellular nucleases by retaining the "ends" of a ssDNA in double stranded form.

It will also be evident to those skilled in the art from this description of the preferred embodiments of the invention that the stem (duplex DNA) can be designed to contain a predetermined sequence (or sequences), i.e., aptamers, that are recognized and bound by specific DNA-binding proteins. Among other uses, such a stem structure is used in the cell as a competitor to titer out a selected protein(s) that regulate specific gene function. For example, a ss-cDNA stem-loop is produced in accordance with the present invention in a cell that contains a binding site for a selected positive transcription factor such as adenovirus E1a. Adenovirus E1a, like other oncogenes, modulates expression of several adenoviral and cellular genes by affecting the activity of cell-encoded transcription factors, resulting in the changing of normal cells to transformed cells. Jones, *et al.*, 2 Genes Dev. 267-281 (1988). The duplex stem of the stem-loop intermediate produced in accordance with the present invention thus functions to "bind up" the factor, preventing the protein from binding a promoter and thus inhibiting expression of a particular deleterious gene. To those skilled in the art, it will be clear that the duplex stem structure may optionally contain multiple binding sites, for example, sites which are recognized by various transcription factors that actively regulate expression of particular gene. For

example, adenovirus E1a has been found to repress transcription of the collagenase gene via the phorbol ester-responsive element, a promoter element responsible for the induction of transcription by 12-O-tetradecanolyphorbol 13-acetate (TPA), by a number of other mitogens, and by the *ras*, *mos*, *src*, and *trk* oncogenes. The mechanism involves inhibition of the function of the transcription factor family AP-1. (Offringa, *et al.*, 62 Cell 527-538 (1990)). Any desired nucleotide sequence can be inserted into the genetic element which encodes the "loop" portion of the stem-loop intermediate to ultimately carry out a desired inhibitory function, e.g., antisense binding, down regulation of a gene, and so on as herein described.

In another aspect which will be recognized by those skilled in the art, the present invention is used to construct complex secondary ssDNA structures which confer biologic reactions on the cDNA transcript produced in accordance with the present invention based on conformational secondary structure folding. Such secondary structure can be engineered to serve any of several functions. For instance, the sequence of interest may include (but is not limited to) a sequence which is incorporated into the loop portion of the single-stranded cDNA transcript which forms so-called "clover leaf" or "crucible" like structures such as those found in the long terminal repeats of adeno-associated virus or in retrotransposons. Under correct circumstances, such structure is integrated in site-specific manner into the host genome.

Because the cassette of the present invention is adaptable for incorporation into multiple commercially available delivery vectors for mammalian and human therapeutic purposes, multiple delivery routes are feasible depending upon the vector chosen for a particular target cell. For example, viral vectors are presently the most frequently used means for transforming the patient's cells and introducing DNA into the genome. In an indirect method, viral vectors carrying new genetic information, are used to infect target cells removed from the body, and these cells are then re-implanted (i.e., *ex vivo*). Direct *in vivo* gene transfer into postnatal animals has been reported for formulations of DNA encapsulated in liposomes and DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Nicolau *et al.*, 80 Proc. Natl. Acad Sci USA 1068-1072 (1983); Kaneda *et al.*, 243 Science 375-378 (1989); Mannino *et al.*, 6 Biotechniques 682-690 (1988)). Positive results have also been described with calcium phosphate co-

precipitated DNA (Benvenisty and Reshef, 83 Proc. Natl. Acad. Sci. USA 9551-9555 (1986)). Such systems include intravenous, intramuscular, and subcutaneous injection, as well as direct intra-tumoral and intra-cavitary injections. The cassette, when inserted into the vector of choice is also advantageously administered through topical, transmucosal, rectal, oral, or inhalation-type methods of delivery.

The cassette of the present invention is advantageously employed to deliver anti-sense, triplex, or any other single-stranded inhibitory nucleic acid sequence of interest, using known digestion and ligation techniques to splice the particular sequence of interest into the cassette between the inverted tandem repeat, between the inverted tandem repeat and the 3' primer binding site, or in both locations. Those skilled in the art who have the benefit of this disclosure will also recognize that the above-described signals used for expression within eukaryotic cells may be modified in ways known in the art depending upon the particular sequence of interest. The most likely change is to change the promoter so as to confer advantageous expression characteristics on the cassette in the system in which it is desired to express the sequence of interest. There are so many possible promoters and other signals, and they are so dependent on the particular target cell for which the sequence of interest has been selected, that it is impossible to list all the potential enhancers, inducible and constitutive promoter systems, and/or poly(A) tailing systems which may be preferred for a particular target cell and sequence of interest.

In one particularly preferred embodiment, the present invention takes the form of a kit comprised of a plasmid having the above-described RNA-dependent DNA polymerase and restriction endonuclease genes cloned therein as well as a multiple cloning site (MCS) into which the user of the kit inserts a particular sequence of interest. The cloning site into which the sequence of interest is inserted is located between the above-described inverted tandem repeats. The resulting plasmid is then purified from the cell culture in which it is maintained, lyophilized or otherwise preserved for packaging and shipping, and sent to the user. The kit preferably also includes the restriction endonuclease for the cloning site into which the sequence of interest is to be cloned, the ligases and other enzymes, along with suitable buffers, for ligating the sequence of interest into the plasmid, and a map of the plasmid.

Except where otherwise indicated, standard techniques as described by Seabrook, *et al.* (1989) (J. Seabrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Ed.), Cold Spring Harbor Press (1989), hereinafter referred to as "Maniatis, *et al.* (1989)") and Ausubel, *et al.* (1987) (F.M. Ausubel, *et al.*, Current Protocols in Molecular Biology, New York: John Wiley & Sons (1987)), both of which are hereby incorporated in their entirety by this specific reference thereto, were utilized in the examples set out below. It should be understood that other methods of production of ssDNA, both by natural processes and by designed artificial methods using different enzyme products or systems, may also be utilized in connection with the method of the present invention and that the example set out herein are set out for purposes of exemplification as required by the Patent Statute and do not limit the intended scope of this disclosure.

The plasmid pcDNA3.1/Zeo+ was purchased from Invitrogen Corp. (Carlsbad, CA) and plasmid pBK-RSV from Statagene (La Jolla, CA). Oligodeoxynucleotides (ODN) were synthesized by Midland Certified Reagent Co. (Midland, TX). Polymerase chain reactions (PCR) were carried out using Taq DNA polymerase purchased from Boehringer Mannheim Corp. (Indianapolis, IN) in a Robo-gradient thermal cycler (Stratagene (La Jolla, CA). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). The ODNs used are listed in the attached Sequence Listing.

EXAMPLE 1. *In vivo* Synthesis of ss-cDNA in Eukaryotic Cells

The following *in vivo* experiments were designed to test whether the plasmids made in accordance with the present invention ssDNA in eukaryotic tissue culture cells.

Plasmid constructs. Except as otherwise noted, the ODNs were allowed to hybridize in 1 µl (5 µg/µl in water) in four separate tubes which were incubated at 70°C for 5 min and allowed to hybridize for 15 min at room temperature. Standard restriction endonuclease digests were carried out (EcoR I used as a negative control) with 10 units of enzyme in a total reaction volume of 15 µl and appropriate reaction buffers. DNA fragments were resolved in and isolated from agarose gels. The selection of positive clones on ampicillin plates was performed after transformation into competent XL1-Blue MRF' cells (Stratagene) as described by Maniatis, *et al.* (1989) and the accompanying

instruction. After positive clones were picked, plasmid DNA was isolated using a commercially available plasmid isolation kit (Quiagen, Inc., Sant Caita, CA). Confirmation of DNA ligation was carried out by 0DNA sequencing.

Construction of B Plasmid. A first embodiment of one of the two plasmids comprising the two plasmid embodiment of the vector system of the present invention is the "4B" plasmid. The 4B plasmid, like plasmid pssDNA-Express-B described in my co-pending application Serial No. 09/397,782 (which application is hereby incorporated into this application in its entirety by this specific reference), was derived from pcDNA3.1/Zeo+ (Invitrogen Corp.), shown in Fig. 4A. pssDNA-Express-4B was constructed by digesting with restriction endonucleases Hind III and Not I at positions 911 and 978, respectively. The double-stranded linker region having compatible Hind III and Not I ends which is formed by annealing the synthetic, single stranded oligodeoxynucleotides ODN-5'-N/M(link)2-H/N and ODN-3'-N/M(link)2-H/N was ligated under standard conditions into the digested pcDNA3.1/Zeo+ transformed into Sure II cells (Stratgene, Inc.). The ODNs were allowed to hybridize in 1 μl (5 μg/μl in water) in Ependorf tubes that were incubated at 70°C for 5 minutes and allowed to hybridize for 15 minutes at room temperature. Appropriate clones were selected and sequenced to assure proper insertion of the linker region. The resulting plasmid was termed pcDNA3.1/Zeo+/NM-link2-gag and re-named pssDNA-Express-4B. pssDNA-Express-4B is shown in Fig. 4B and is the plasmid into which the sequence of interest is cloned (Fig. 4C). For cloning sequences of interest between the inverted tandem repeats, the two Not I sites as positions 935 and 978, respectively (see Fig. 4D), were used. These two sites are contained within the inverted tandem repeats. For inserting sequences of interest between the inverted tandem repeats and the primer binding site, two convenient restriction endonuclease sites, Pac I and Bam H I, at positions 1004 and 1021, respectively, were used.

Construction of A Plasmid. The second plasmid of the two plasmid system which comprises one embodiment of the vector of the present invention is the A plasmid. This plasmid, designated pssDNA-Express-A, is shown in Fig. 3A. pssDNA-Express-A contains the MoMuLV-RT (T.M. Shinnick, *et al.*, 293 Nature 543-548 (1981)) and

restriction endonuclease genes and was derived from pBK-RSV (Stratagene), also using XL-1 Blue MRF' as the host strain. A mouse cell line expressing Moloney murine leukemia virus was obtained from the American Type Culture Collection (#CRL-1858). The virus RNA was isolated and prepared for reverse transcriptase-PCR (RT-PCR). A 2.4 kb fragment containing the coding sequence of MoMuLV-RT was PCR-amplified using primers as set out in Seq. ID 1/ODN-RT(-) (primer position at nucleotide #2545) and Seq. ID 2/ODN-RT(+) (primer position at nucleotide #4908) to produce a DNA fragment with a 5'-Sac I and a 3'-Hind III compatible end. The 2.4 kb product obtained includes the sequence of the MoMuLV genome between positions 2546 and 4908. The mature virus reverse transcriptase peptide is encoded by the sequence between positions 2337 and 4349 (Petropoulos, C.J., Retroviral taxonomy, protein structure, sequences and genetic maps, *in* J.M Coffin (Ed.), Retroviruses, 757, Appendix 2, New York: Cold Spring Harbor Press (1997)), but peptides truncated at the amino terminus retain full activity (N. Tanese, *et al.*, 85 Proc. Natl. Acad. Sci. USA 1777-1781 (1998)). The peptide encoded by this construct includes a part of the integrase gene, which follows the reverse transcriptase in the MoMuLV polyprotein, but is not relevant here such that the length of the construct was selected because of the availability of a convenient restriction site for cloning.

The bacterium *Moraxella bovis*, which encodes the restriction endonuclease MboII (H. Bocklage, *et al.*, 19 Nucleic Acids Res. 1007-1013 (1991)), was obtained from the American Type Culture Collection (ATCC#10900). Genomic DNA was isolated from *M. bovis* and the *Mbo* II gene was used as the template DNA in the PCR. A 1.2kb fragment containing the *Mbo* II gene was amplified by PCR using as primers Seq. ID 3/ODN-Mbo(+) (primer position at nucleotide #887) and Seq. ID 8/ODN-Mbo(-) (primer position at nucleotide #2206). These primers contain mismatches designed to introduce a a *Hind* III site into the 5' primer and an *Xba* I site into the 3' downstream primer. The 1.2kb DNA amplification product, copying the *M. bovis* genome between positions 888 and 2206, therefore contains the coding region for the Mbo II protein. The amplification product was digested with Hind III and Xba I.

The pBK-RSV vector was digested with Xba I and Nhe I, which removes the promoter region. The *Nhe* I end was converted to a *Sac* I end using the linker formed by

annealed oligodeoxynucleotides Seq. ID 6/ODN-N>S(+) and Seq. ID 7/ODN-N>S(-). The reverse transcriptase and Mbo II amplimers were ligated through the Hind III sites and this construct was subsequently ligated between the *Sac* I and *Xba* I sites of pBK-RSV to produce pBK-RSV-RT/Mbo.

To insert a flexible linker between the reverse transcriptase and Mbo II domains of the polyprotein and to provide a tag useful for purification of the protein, the double-stranded sequence formed by annealing the oligodeoxynucleotides Seq. ID 9/ODN-HisPro(+) and Seq. ID 10/ODN-HisPro(-), encoding alternate histidine and proline amino acids, was ligated into pBK-RSV-RT/Mbo. pBK-RSV-RT/Mbo was digested with Hind III, and the his-pro linker, with compatible *Hind* III ends, was inserted at the *Hind* III site to produce plasmid pBK-RSV-RT/Mbo-L and the orientation was confirmed by sequencing.

Sequencing pBK-RSV-RT/Mbo-L revealed a frame shift mutation at the 5'-end of the Mbo II domain. This mutation was corrected, and the extraneous part of the integrase gene of MoMuLV was removed simultaneously, by excising the fragment of the plasmid lying between the *Ase* I and *Bgl* II sites, which encodes the 5'-end of the *Mbo* II gene, the his-pro linker region, and the integrase gene fragment and replacing with an insert containing a modified his-pro linker and 5'-*Mbo* II gene fragment. The modified his-pro linker increased the number of histidines by one, to six, and included at the 5'-end a number of unique restriction sites. The 5'-end of the Mbo II gene was modified to replace the leucine at the N-terminus that was introduced by the mismatch in the PCR primer to the original methionine and to optimize codon usage for expression of this segment of the gene in mammalian cells. The repair construct was obtained by mutually-primed DNA synthesis from two templates, ODN-Rep(+) and ODN-Rep(-), that have complementary sequences of 16 bases at the 3'-ends. These oligodeoxynucleotides were annealed and extended with the modified SEQUENASE™ DNA polymerase enzyme (United States Biochemical Corp.). The double-stranded product was digested with Ase I and Bgl II and inserted into the vector to give the plasmid pssDNA-Express-A (plasmid A).

The structure of pssDNA-Express-A is shown in Fig. 3A. As set out above, to construct this plasmid, sequences encoding an active fragment of the MoMuLV reverse transcriptase and the *M. bovis* Mbo II restriction enzyme were cloned between the *Nhe* I

and *Xma* I sites of the eukaryotic expression vector pBK-RSV. Transcription of the cloned region is driven by the RSV promoter and selection for transformed cells is carried out in the presence of the antibiotic G418 (neomycin). Reverse transcriptase and Mbo II are expressed as a single, bifunctional protein chain with the two functional domains separated by a short, histidine and proline rich linker.

Construction of C Plasmid. To produce the C plasmid, plasmid pssDNA-Express-A was digested with Sac I Xma I to remove the Mbo II gene (Fig. 3B). A linker region comprised of oligonucleotides 5'-(link)2-Hind/Xba and 3'-(link)2-Hind/Xba, which were allowed to anneal at 70°C for 15 minutes and slowly cooled to room temperature, was ligated into the plasmid after digestion under standard conditions. Positive clones were harvested and sequenced to verify linker placement and this plasmid was then digested with Xba and Hind III. The plasmid pssDNA-Express-B was then digested with Hind III and Xba and the corresponding 300 base pair DNA fragement containing the previously described inverted tandem repeats, multiple cloning site, and PBS was cloned into the digested plasmid to give pssDNA-Express-C (Fig. 5A). Standard ligation reactions were performed and transformed into Sure II cells (Stratagene, Inc.). Transformed positive colonies were harvested and positive clones were identified by restriction analysis.

The sequences of interest were cloned into the multiple cloning site of pssDNA-Express-C by using the Bam H I and Pac I sites in the multiple cloning site (Fig. 5B). Four different sequences of interest as listed in Table 1, each including the "10-23 DNA enzyme" inserted between the 5' and 3' aspects of the antisense sequence (Fig. 5C), were synthesized for these constructs, and similar procedures were utilized for inserting each of the four sequences of interest. Each construct was prepared by allowing the paired oligonucleotides to anneal at 70°C for 15 minutes and cooling to room temperature, followed by ligation into the plasmid under standard conditions. After transformation into Sure II cells, appropriate colonies were selected with verification by sequencing for the individual inserts. The antisense ability of each of these plasmids was tested by transfecting each plasmid, with appropriate controls which contained a random sequence of equal length and did not contain antisense inserts or the "10-23 DNA enzyme," into HeLa cells using lipofectant reagent (Boehringer Mannheim) under standard conditions.

Trizol reagent was used to harvest the cells and RNA fraction, and subsequent Northern blot analysis was performed to demonstrate specific antisense expression.

Tissue culture studies. Stable and transient transfections were carried out by using lipofectant (Boehringer Mannhiem Corp.) using the manufacturer's accompanying instructions. All plasmid constructs were transfected into HeLa cell lines. Assays for ssDNA were performed by PCR and by dot-blot analyses 24-48 hours after transfection. Reverse transcriptase activity was assayed using the RT-PCR assay developed by Silver, et al. (Silver, J., *et al.* 21 Nucleic Acids Res. 3593-4 (1993)) after transfection with pssDNA-Express-A plasmid. Individual colony isolates of stably substituted HeLa cell lines (A12 and B12) were additionally assayed for RT activity. The ss-cDNA was isolated from cells transfected 48-72-hr earlier. The ss-cDNA, which co-localizes with RNA, was carried out using trizol reagent (Gibco Life Technologies, Gaithersburg, MD). Assays for specific ss-cDNA species were carried out by both PCR based assays for internal fragment and by denatured single stranded gel electrophoresis with subsequent nylon blotting and probing with an internal biotin-labeled probe.

This experiment showed that human tissue culture cells (HeLa cell line), transfected with plasmids designed to synthesize a processed ss-cDNA, produced ss-cDNA of the predicted size. As described in the above-incorporated application Serial No. 09/397,782, the ssDNA sequence of interest produced in accordance with the method of the present invention is produced from either the position between the inverted repeats after digestion of the stem of the stem-loop intermediate or from the position between the inverted repeats and the primer binding site by premature termination of the reverse transcriptase cDNA transcript at the 3' aspect of the stem structure. The sequence of interest produced from this premature termination is the second sequence of interest referred to herein. Fig. 7 shows that cells transfected with plasmid pssDNA-Express-4B having the 10-23 enzyme included in the antisense sequence against c-raf kinase which was utilized as the sequence of interest produced an antisense sequence against c-raf kinase that included the 10-23 DNA enzyme from the position between the inverted tandem repeats and the primer binding site.

* * * * *

The experiments described above demonstrate two methods for producing ssDNA including enzymatic sequences *in vivo* by multiple stepwise reactions using eukaryotic reverse transcriptase reactions and various cDNA priming reactions. These reactions were followed by formation of a "stem-loop" intermediate which can be used to eliminate any unwanted sequences either (a) upstream 5' or downstream 3' from a designed (and formed) "stem" after being subsequently cleaved by a restriction endonuclease or (b) by premature termination of the ss-cDNA from a 3' sequence of interest.

Although described with reference to the figures and specific examples set out herein, those skilled in the art will recognize that certain changes can be made to the specific elements set out herein without changing the manner in which those elements function to achieve their intended respective results. For instance, the cassette described herein is described as comprising a sequence of interest including an enzymatic sequence and a tandem inverted repeat. Any sequence (or sequences) of interest including an enzymatic sequence is produced by these methods in any eukaryotic cell. The sequence(s) of interest is cloned (or synthesized) between the designed inverted tandem repeats and represents the sequence in the "loop" after ssDNA production and subsequent stem-loop formation. In addition (or instead of), the sequence(s) of interest including the enzymatic sequence is cloned (or synthesized) 3' to the inverted repeats. The sequence(s) of interest to be produced are comprised of any base (i.e., A,T,G,C) composition as long as the sequence does not interfere with the formation of the stem of the stable stem-loop intermediate. The sequence having enzymatic activity (when in the form of ss-DNA) can be any of the DNAse, RNAse, DNA kinase, and/or DNA ligases demonstrated to have such functions.

Those skilled in the art will likewise recognize that, for instance, the mouse Moloney leukemia virus reverse transcriptase gene described for use as the reverse transcriptase gene of the cassette can be replaced with other reverse transcriptase genes (the reverse transcriptase gene from Human immunodeficiency virus was one such gene which was noted above) and that promoters other than the CMV promoter may be used to advantage. Further, several restriction endonuclease genes are listed above, but those skilled in the art will recognize from this description that the list set out above is not exhaustive and that many other restriction endonuclease genes will function to advantage

in connection with the present invention. Similarly, the RSV promoter described as being used in connection with the restriction endonuclease genes set out herein is not the only promoter which may be used to advantage. All such changes and modifications which do not depart from the spirit of the present invention are intended to fall within the scope of the following non-limiting claims.

Table #1

Oligodeoxynucleotides (ODN's)

Name: 5'-N/M(link)2-H/N
5'AGCTTGGTCGGCGGCCTTGAAGAGCGGCCGCACTCACGATAGAGTGGGAGATGGGCGCGAGAAAGTGCGGCC
GCTCTTCAAGGCCGCCGACCTTAATTAAGTCAGCGGGGATCCTTTTTGGGGGCTCGTCCGGGATCGGGAGACC
CCT-3'

Name: 3'- N/M(link)2-H/N
5'GGCCAGGGGTCTCCCGATCCCGGACGAGCCCCCAAAAAGGATCCCCGCTGACTTAATTAAGGTCGGCGGCCT
TGAAGAGCGGCCGCACTTTCTCGCGCCCATCTCCCACTCTATCGTGAGTGCGGCCGCTCTTCAAGGCCGCCGACC
A-3'

Name: 5'- polyNM-gaglink-(Pleio)-DNAse-1023-B/P
5'-GAT GTA AG TCG TTG TAG CTA GCC TCC CCT G -3'

Name: 3'-polyNM-gaglink-(Pleio)-DNAse-1023-B/P
5'-GAT CCA GGG GA GGC TAG CTA CAA CGA CTT ACA TCA T -3'

Name: 5'-polyNM-gaglink-(hras)-DNAse-1023-B/P
5'-GGTGGG CGCCTCGTTGTAGCTAGCCTCGGTGTGGG-3'

Name: 3'- polyNM-gaglink-(hras)-DNAse-1023-B/P
5'-GATCCCCACACCGAGGCTAGCTACAACGAGGCGCCCACCAT-3'

Name: 5'-polyNM-gaglink-(rafK)-DNAse-1023-B/P
5'-AATGCATGTCTCGTTGTAGCTAGCCCAGGCGGGA-3

Name: 3'- polyNM-gaglink-(rafK)-DNAse-1023-B/P
5'-GATCTCCCGCCTGGGCTAGCTACAACGAGACATGCATTAT-3'

Name: 5'-polyN/M-gaglink-(tat-SIV)-DNAse-1023-B/P
5'-AGATGGAGACTCGTTGTAGCTAGCCCCCTTGAGGGCAGATTGGCGCCCGAACAGGGACTTGAAGGA-3'

Name: 3'- polyN/M-gaglink-(tat-SIV)-DNAse-1023-B/P
5'-GATCTCCTTCAAGTCCCTGTTCGGGCGCCAATCTGCCCTCAAGGGGGCTAGCTACAACGAGTCTCCATCTAT-3'

Name: 5'-(LINK)2-Hind/Xba
5'-CCG GAT CTA GAC CGC AAG CTT CAC CGC -3'

Name: 3'-(LINK)2-Hind/Xba
5'-GGT GAA GCT TGC GGT CTA GAT -3'

Table #1 (CONT'D)

ODN-RT (+) 32 bases (#13)	5′-GGGATCAGGAGCTCAGATCATGGGACCAATGG-3′
ODN-RT (-) 24 bases (#12)	5′-CTTGTGCACAAGCTTTGCAGGTCT-3′
ODN-N>S (+) 18 bases (#25)	5′-CTAGCGGCAAGCGTAGCT-3′
ODN-N>S (-) 10 bases (#26)	5′-ACGCTTGCCG-3′
ODN-Mbo (+) 30 bases (#16)	5′-CAATTAAGGAAAGCTTTGAAAAATTATGTC-3′
ODN-Mbo (-) 27 bases (#33)	5′-TAATGGCCCGGGCATAGTCGGGTAGGG-3′
ODN-HisPro (+) 43 bases (#36)	5′-AGCTGGATCCCCCGCTCCCCACCACCACCACCACCCTGCCCCT-3′
ODN-HisPro (-) 42 bases (#37)	5′-AGCAGGGGCAGGGTGGTGGTGGTGGTGGGGAGCGGGGGATCC-3′
ODN-Rep(+) 121 bases	5′-ATATCTATTAATTTTGGCAAATCATAGCGGTTATGCTGACTCAGGT GAATGCCGCGATAATTTTCAGATTGCAATCTTTCATCAATGAATTTCAG TGATGAATTGCCAAGATTGATGTTGC-3′
ODN-Rep(-) 111 bases	5′-GACGAGATCTCCTCCAGGAATTCTCGAGAATTCGGATCCCCCGCTC CCCACCACCACCACCACCACCCTGCCCCGCGGATGAAAAATTATGTGAG CAACATCAATCTTGGC-3′